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Prostate cancer is the second leading cause of death among American men. The present proposal extends the molecular progress in prostate cancer into clinical practice by developing innovative markers. The current androgen ablation treatment is initially therapeutic, but most patients relapse. A novel androgen substitution approach uses androgens to promote terminal differentiation and arrest cancer. Markers of differentiation, however, are not known in the prostate. We discovered a novel gene, AS3 (new name APRIN), a marker of proliferative arrest and differentiation. We mapped the AS3 gene on chromosome 13 and we found that the loss of the gene correlates with a high incidence of prostate cancer. In many of the cancers genetic markers are not informative, raising the possibility that epigenetic mechanisms contribute to AS3 silencing. The objective of this proposal is to investigate the epigenetic mechanisms to assess AS3 gene silencing in prostate cancer. In the first year we established the technology to isolate cancer specific DNA and worked out the methylation specific PCR and sequencing techniques on the AS3 promoter. We generated the first data that methylation of the APRIN promoter is critical in the silencing of the gene and correlates with hormone resistance in the LNCaP-TJA prostate cancer cell line. These data have been confirmed by direct sequencing of the bisulfite-converted amplified promoter elements. In addition, these data identified methylation hot-spots in the promoter of the APRIN gene. Our results established a critical set of reagents and experience for the tasks of the second year.

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Table of Contents

INTRODUCTION

The statistics published every year by the American Cancer Society indicate that prostate cancer continues to increase in the United States. We know more about the molecular processes than ever before, but the application of this knowledge in the clinical care is practically non-existent.

The goal of this proposal is to establish a technology which will allow highly sophisticated molecular information to be directly applied into clinical practice. The methods we propose can detect gene silencing using a novel approach at the level of the promoter. When the gene is silenced, it is blocked by methylation and we can detect this modification (hypermethylation).

The gene we target and the methods we propose may be able to have a significant impact on the diagnosis and therapy of prostate cancer. Our previous work has introduced novel findings into this field of prostate cancer research. We investigated the mechanisms of terminal differentiation in the prostate and identified a key regulator of the process, the AS3 (new name APRIN) gene. Expression of the gene indicates differentiation in animals and brings about proliferative arrest in cell culture. We also found that AS3 is frequently lost in prostate and other cancers, strongly suggesting that it is also a tumor suppressor.

We hypothesize that the status of AS3 is critical in the prognosis, the therapy and the outcome of prostate cancer. The presence of AS3 indicates that differentiation is functional in these cancers and that hormonal therapy will benefit the patients. The information, therefore, on the status of AS3 may be the most important variable to make therapy decisions.

The studies we proposed will supply that information in an extremely sensitive, specific and simple way. A line of evidence indicates that APRIN is silenced in many cancers. We investigate if the silencing is the result of DNA methylation in the promoter. The methods are based on the amplification of modified DNA which reflects the methylation status. Since the modification we test (promoter methylation) is absent from normal cells, any positive signal indicates disregulation and may serve as the earliest sign of malignant cells in the body.

In the first year of the project we established the basic reagents to perform further clinical studies. Using this new set of reagents we generated the first data that DNA methylation takes place in the APRIN promoter and established a preliminary correlation with promoter methylation and APRIN silencing.

RESULTS

TASK 1: (i) To establish the methodology to isolate DNA from various biological sources and (ii) to use the isolated DNA to evaluate methylation-specific techniques on the AS3 gene. (These activities are expected to continue to a lesser extent during the second year, depending on the availability of the clinical samples and archival sources) (Months 1-12)

1)/ DNA extraction methods from postoperative tissues (Months 1-9).

To establish the optimal procedures for the isolation of high molecular weight DNA from frozen tissues, we decided that the learning curve and the inevitable errors and waste should not be done on highly valuable human samples. The first experiments were performed on a material of easier access, on frozen rat prostates. We tried the QIA AMP DNA kit, the DNeasy Tissue Kit (both from Qiagen) and the Wizard SV DNA Purification System from Promega. We used 20-200 mg tissue samples and followed the manufacturers instructions and modifications from the literature (1). All of the systems generated high molecular weight (by agarose gel electrophoresis) and high quality DNA preparations (260/280 ratios were better than 1.8).

Our access to human samples were limited in the first year. The Human Subjects Research Review Board of the US Army Medical Research and Materiel Command found several formal mistakes in the human materials approval by the local IRB at Tufts University. After reformulating and re-certifying the necessary forms, we received the final permission from the Human Subjects Research Review Board of the US Army Medical Research and Materiel Command in the end of November, 2003. By this time, our collaborator at the Urologic Oncology Department of the Tufts-New England Medical Center, Dr John Long left the institution. By March 2004 we succeeded in finding another surgical expert at the same institution, Dr Gennaro A. Carpinito. Since we changed a key collaborator, both the local IRB and the Human Subjects Research Review Board of the US Army Medical Research and Materiel Command required us to re-certificate our human material projects. The procedure is in progress and in the second year of the project we will be cleared to have access for postoperative human prostate and blood samples. Under these conditions we concentrated our efforts to work out the molecular aspects of the project, to design and fine tune the methylation detection methodology.

2) *DNA extraction methods from paraffin-embedded archive materials using Laser Capture Microdissection for initial DNA quality analysis (Months 1-9).* We received a series of laser caption microdissected samples from our other collaborator, Dr Irwin Leav. The 10 samples represent normal periferal zone, benign prostatic hypertrophy (BPH) stroma and gland samples, moderate prostatic intraepithelial neoplasma (PIN) samples, carcinoma samples from grade 3, grade 4/5 and intraductal carcinoma samples. To extract DNA from the samples we used the PicoPure DNA Extraction Kit from Arcturus and the DNeasy Tissue Kit from Qiagen. The DNA preparations were tested using AS3 promoter-specific primers and we could show good quality detectable DNA from the micro-samples of laser caption microdissection. The results and the primer sets will be discussed in Section 6 of the report.

3) DNA extraction methods from blood (3 samples from 3 of the above 10 postoperative patients) and needle biopsy materials (Months 1-9). We did not have access for patient blood samples in the first year of the project, for reasons explained in Section 1 of the report. We had access, however, for human needle biopsy samples from prostate cancer through commercial sources. These de-identified and exempt commercial products contain needle biopsy sample sections from 20 to 70 patients with detailed diagnoses

from expert pathologists. We purchased the needle biopsy samples in tissue array format from the Cybrdi Company (Catalog number CC19-01-003). To extract the DNA from the tissue sample, we used the EX-WAX DNA Extraction Kit from the Chemicon company, following the instructions for the kit. We tested the DNA preparations by AS3 promoter-specific primers in PCR reactions. We detected amplifyable DNA, the starting material to our methylation studies. The results and the primer sets will be discussed in Section 6 of the report.

4) Perform chromatin immune precipitation studies (ChIP) with the MeCP2 protein to study the overall methylation status of the AS3 promoter (from the above 10 postoperative samples and from cell lines LNCaP and MCF7) (month 6-12). Chromatin immune precipitation is a specific and highly sensitive method to detect protein-DNA complexes. The principle of the method is non-saturating formaldehyde crosslinking of protein-DNA complexes, which are isolated by specific antibodies through immune precipitation. The precipitate is purified for DNA and PCR reactions with target gene specific primers detect the involvement of the gene in the protein complex. We followed the procedure by Strahl-Bolsinger et al. (2). We used LNCaP-TJA cells, a prostate cancer cell line, which was derived from the LNCaP-FGC cells (3). While the LNCaP-FGC line responds to androgen by the induction of the AS3 protein and proliferative arrest, the TJA line lost this ability. Since AS3 is not expressed, we assumed that AS3 is silenced by epigenetic, promoter-methylation mechanisms. TJA cells were grown in T25 flasks for confluency, trypsinized and treated with 1% formaldehyde in PBS at room temperature for 15 min. The cells and their DNA were fragmented by 30 sec sonication and the supernatant was used for immune precipitation by the anti-MeCP2 antibody (Methyl-CpG-Binding Protein 2) from Upstate Biothechnology and Protein A-Sepharose from Sigma, following standard procedures. After several washes in 1% Triton and 500 mM NaCl in PBS and finally in 250 mM LiCl , 0.5% NP-40, 0.5% Na Deoxycholate in PBS, the specific complex was eluted by 1% SDS in TBS at 65C^{o} overnight. The DNA was extracted by using the Qiagen PCR purification kit and eluted in 50 ul elution buffer. We detected the AS3 promoter in the precipitated material using AS3-specific primers (for details see Section 6). The presence of the promoter in the MeCP2 complex indicates that AS3 is silenced in the LNCaP-TJA cells by promoter methylation mechanisms.

5) Perform methylation-sensitive restriction enzyme analysis by Southern blots to target particular methylation sites (from 3 post-operative samples, 5 archive materials and from cell lines LNCaP and MCF7) (months 6-12). We found that the HpaI restriction endonuclease isolates a major portion of the AS3 promoter-associated CpG island (from position -435 through position +145). We also found more than 20 methylation-sensitive restriction enzyme sites within this fragment. The Southern blots-based methylation-sensitive restriction enzyme analysis, however, is extremely labor-intense and for the increased sensitivity (single copy promoter) we need to use P32 isotope labeling. In addition, the HpaI fragment is highly GC-rich with long CG repeats. In our preliminary experiments, where we used it as a probe, it generated high non-specific background. So pilot experiments and fine-tuning is a necessity. For all of the above reasons, instead of experimenting with all of the enzymes in a trial-error approach, we will use a targeted strategy. We identified highly methylated CpG units in the AS3 promoter (see below) using methylation-specific PCR methodology. Based on this information, we will apply the methylation-sensitive restriction enzyme analysis as a secondary confirmation assay to verify the methylation data.

6) Synthesize, optimize and evaluate various primer pairs for the targeted CpG sequences (from 3 post-operative samples and from cell lines LNCaP and MCF7) (Months 6-18).

We discovered that the promoter of APRIN (AS3) is a typical "CpG island", it contains an unusual 105 copies of the CG dinucleotide in the CpG island surrounding the first exon of APRIN (Figure 1) (4). This sequence is the target of promoter methylation, which is known to silence other suppressors in cancer (5, 6).

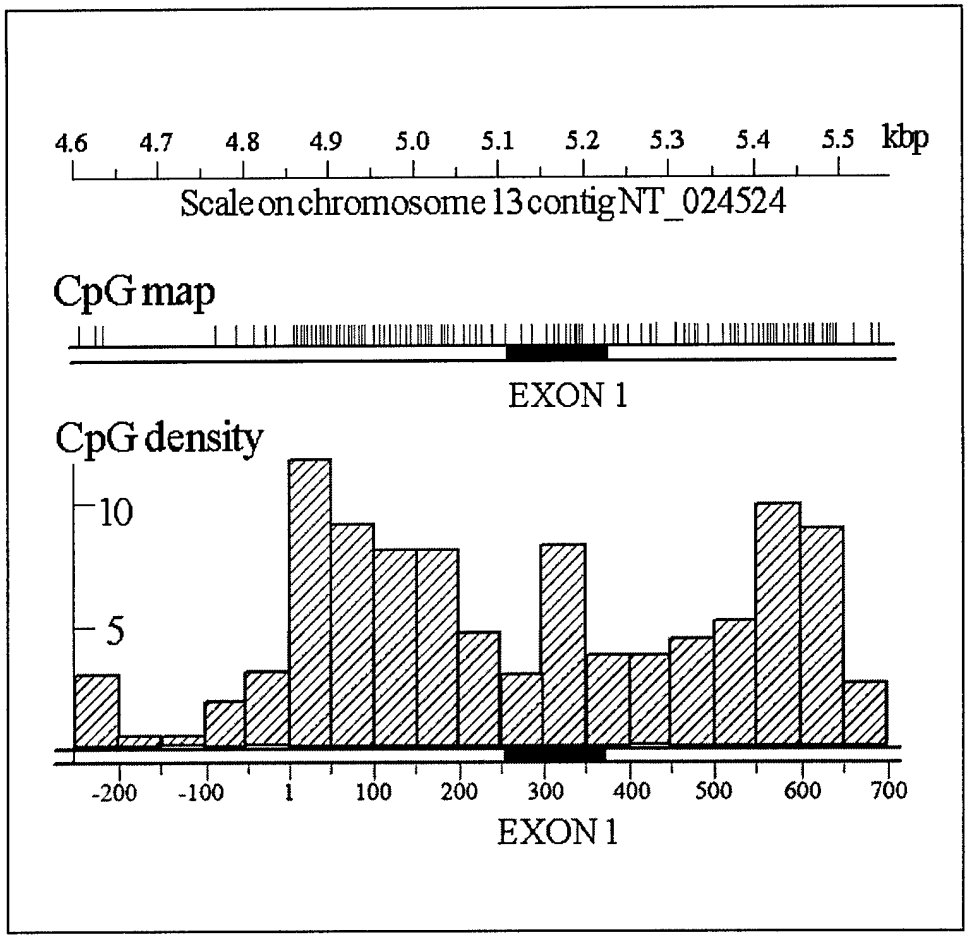

Figure 1 The CpG island of the APRIN (AS3) promoter. The vertical red lines in the CpG map indicate CpG units. Exon 1 of the APRIN gene is shown in blue. The CpG density is expressed in percentage (scale at the left). The graph depicts CpG densities in 50 basepair units. The horizontal numbering in the bottom panel starts with the first base of the CpG island and corresponds with the numbering in Figure 2 and with primer and CpG numbers throughout the report.

To generate the reagents for the detection of methylated CpG units, the first problem was the extreme density of the CpG units in the exon 1 area. In the highly GC-rich sequence it is difficult to find primer sites that meet all the criteria of traditional primer design. We used the MethPrimer web-site to design some of the primers and other resources (GenBank) to optimize primer characteristics. Since the DNA extracted from fixed embedded samples is known to be fragmented (average size <200 bp) and the methylation-specific bisulfite conversion further degrades the DNA (7), we selected primers at intervals covering 80-120 basepairs in average. The selected primer sites are shown in Figure 2 in the next page.

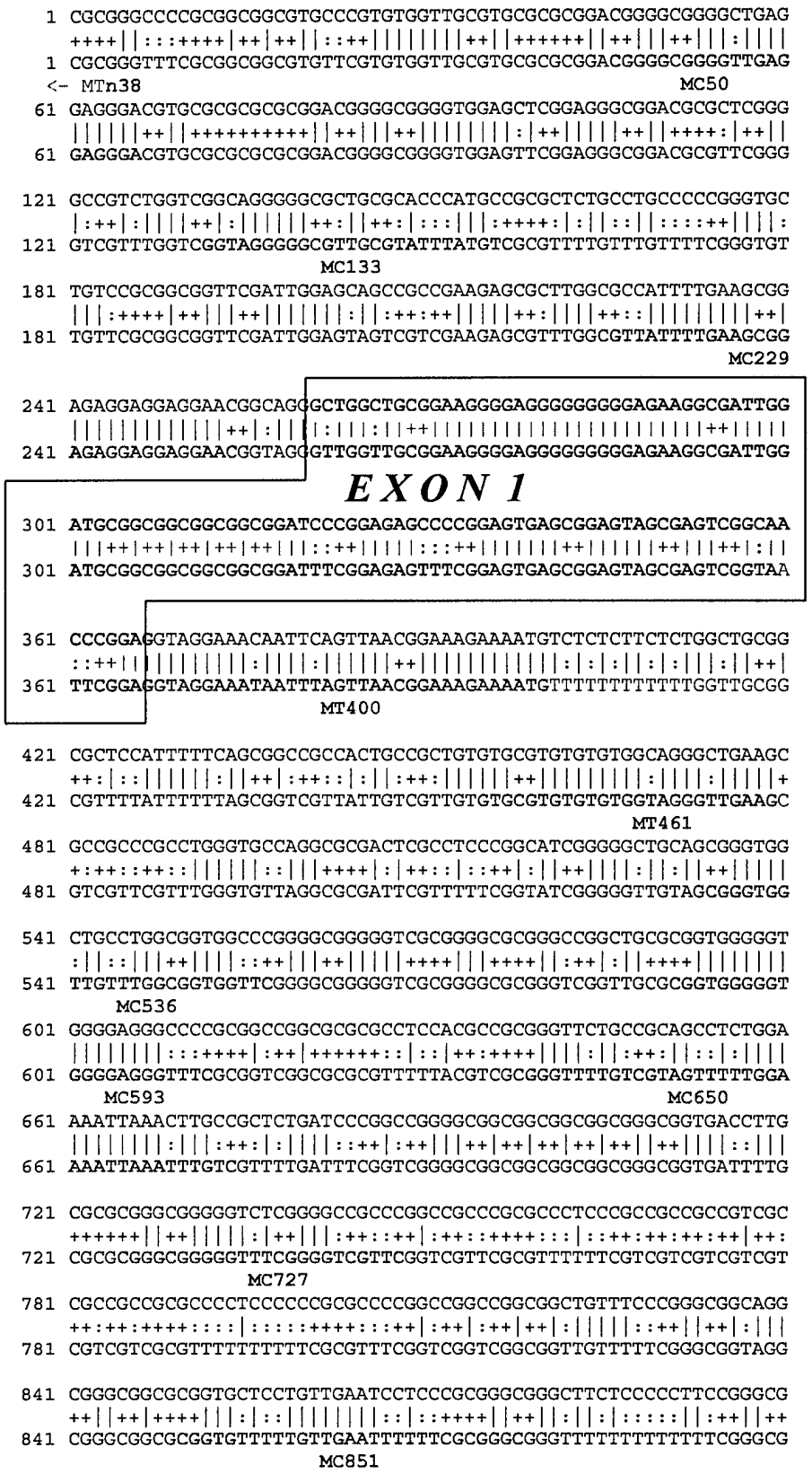

Figure 2 Bisulfite conversion of the APRIN promoter-CpG island sequence. The top sequence represents the native sequence of the APRIN promoter. The bottom sequence

shows the changes induced by the bisulfite conversion of the non-methylated cytidines. The conversion sequence was generated by the MethPrimer program that uses the assumption of full methylation in all the CpG units. The "+" signs indicate CpG units. The " | " signs indicate no change and the " : " signs indicate C to T conversion of the unmethylated C-s. The exon 1 sequence is boxed and is in blue. The primer sequences are in red and bold and their names are also indicated in red

We generated three sets of primers. All of the primers are based on the highlighted areas in Figure 2, but they differ in some important details. The first set of primers (MX series) targets native DNA (the top strand sequence in Figure 2) and serves the purpose of the detection of native DNA to test yields after DNA isolations from minimal samples. The second set (MC series) detects methylated CpG units, while the third set (MT series) amplifies bisulfite-converted DNA for sequencing. Table 1 demonstrates the MX primer series below. The last letter in the names of the primers indicates direction, "a" is for "ahead" or forward; "b" is for "backward" or reverse. The "n" in MXn38a stands for "negative", it is 38 bp upstream of the sequence depicted in Figure 2.

TABLE 1
APRIN promoter primers, to amplify native sequences

Mxn38a	5' gacgccttgacagctgccct	Tm 68
Mx50a	5'gcggggctgaggaggga	Tm 68
Mx50b	5'tccctcctcagccccrc	Tm 68
Mx133a	5'ggcgctgcgtacccatgc	Tm 68
Mx133b	5'gcatgggtacgcagcgcc	Tm 68
Mx229a	5' cattttgaagcggagaggaggagg	Tm 68
Mx229b	5'cctcctcctctccgcttcaaaatg	Tm 68
Mx400a	5'gtaggaaacaattcagttaacggaaagaaaatg	Tm 67
Mx400b	5'cattttctttccgttaactgaattgtttcctac	Tm 67
Mx461a	5'gcgtgtgtgtggcagggc	Tm 68
Mx461b	5'gccctgccacacacacgc	Tm 68
Mx536a	5' gtggctgcctggcggtg	Tm 68
Mx536b	5'caccgccaggcagccac	Tm 68
Mx593a	5' tgggggtggggagggc	Tm 68
Mx593b	5'gccctccccaccccca	Tm 68
Mx650a	5'gccgcagcctctggaaaattaaac	Tm 68
Mx650b	5'gtttaattttccagaggctgcggc	Tm 68
Mx727a	5' ggcgggggtctcggg	Tm 67
Mx727b	5'cccgagacccccgcc	Tm 67
Mx851a	5'gtgctcctgttgaatcctcccg	Tm 68
Mx851b	5'cgggaggattcaacaggagcac	Tm 68

We tested the performance of the above primers in PCR reactions where the primers were combined in the following pattern: Mxn38a with Mx50b. Next, Mx50a with Mx133b. Next, Mx133a with MX229b, and so on. In this way the whole area is covered, but the individual PCR units amplify only 100 bp average, to increase the chance for detection in the fragmented, GC-rich template. The template DNA for the test PCR was isolated from the LNCaP-FGC cell line, using the DNeasy Tissue Kit from Qiagen. The PCR reaction was performed using the TripleMaster kit from Eppendorf, for the highly GC-rich template sequence required special additives in the 10xHigh Fidelity buffer portion of the kit. The following PCR protocol was used: 98-3min (denaturation); 94-

30sec, 64-30sec, 72-1min, 5 cycles (touchdown phase); 94-30sec, 59-30sec, 72-30sec, 40 cycles, followed by 72-3min. The products were run in 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The result is shown in Figure 3.

Figure 3

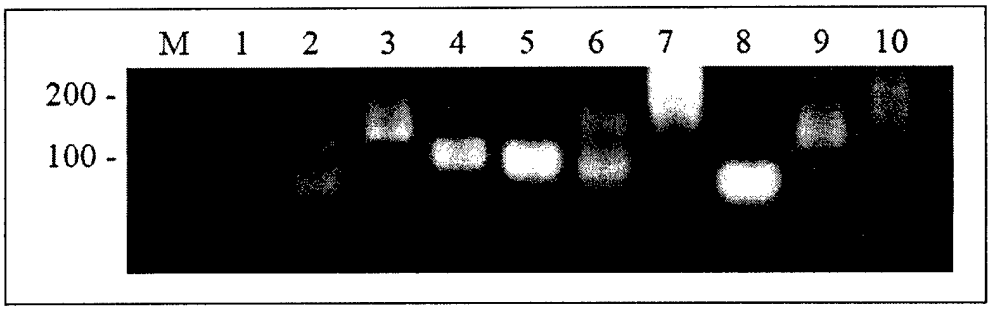

Figure 3. Detection of the native promoter sequence by PCR . The following primer pairs were used: (all MX series) 1, n38a-50b; 2, 50a-133b; 3, 133a-229b; 4, 229a-400b; 5, 400a-461b; 6, 461a-536b; 7, 536a-593b; 8, 593a-650b; 9, 650a-727b; 10, 727a-851b. M, marker, the size range is indicated in basepairs.

The results show that most of the primers worked on the highly GC-rich template. There were some complications (secondary bands, smeary bands, weak bands), but optimization eliminated most of the problems. The important point was that we established the tool set to detect the difficult, high GC target promoter sequence from minimal samples. The primer sets detected the promoter by using the described technology from archived, embedded material (Section 2), from needle biopsy material (Section 3) and from chromatin immune precipitation (Section 5), as shown in Figure 4.

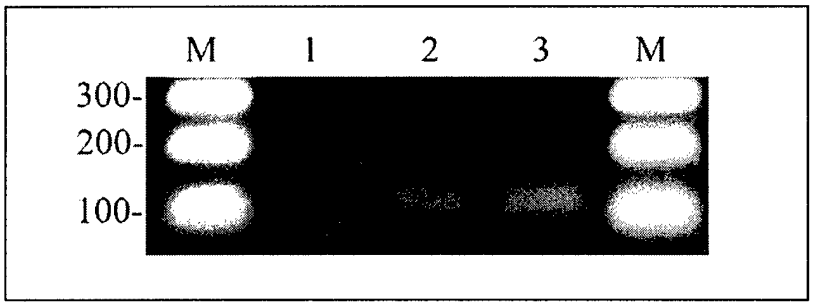

Figure 4. Detection of the APRIN promoter from minimal DNA samples. Lane 1, MX650a-MX851b PCR on DNA from archived, embedded cancer section. Lane 2, MX50a-MX133b PCR on needle biopsy material. Lane 3, MX50a-MX133b PCR on DNA preparation from a chromatin immune precipitation pellet.

7) Work out the best methods for methylation-specific PCR reactions and PCR product analysis (from 3 post-operative samples and from LNCaP and MCF7) (Months 6-18). For large-scale screening analyses we proposed to use the recently developed bisulfite technology to detect methylated CpG sequences in the APRIN (AS3) promoter (8). The treatment changes unmethylated cytosines to thymidines, but methylated cytosines are stable. PCR reactions with primer pairs that target the unchanged (methylated) genomic cytosines detect methylation with great sensitivity. The methylation-specific primer set is shown in Table 2.

TABLE 2
APRIN promoter primers, to target bisulfite-converted, methylated CpG sequences, for MSP analysis

MC50a	5'g**y**ggggttgaggagggac	Tm 60-66
MC50b	5'cctcaaccccc**r**ccccg	Tm 63-68.5
MC133a	5'ggggg**y**gttg**y**gtatttatgtc	Tm 54-63.5
MC133b	5'c**r**caac**r**ccccctaccg	Tm 57.7-68
MC229a	5' tttgaag**y**ggagaggaggaggaac	Tm 64.2-68
MC229b	5'tcctcctctcc**r**cttcaaaataacg	Tm 63.8-67.5
MC536a	5' gtggttgtttgg**y**ggtggttc	Tm 62.3-67
MC536b	5'cc**r**ccaaacaaccacccg	Tm 63-68
MC593a	5' tgggggtggggagggtttc	Tm 68.7
MC593b	5'cctccccacccccaccg	Tm 69.5
MC650a	5'ggttttgt**y**gtagtttttggaaaattaaatttgtc	Tm 66-68
MC650b	5'atttaattttccaaaaactac**r**acaaaacccg	Tm 66.3-68
MC727a	5' g**y**gggggttt**y**ggggt**y**gttc	Tm 64.6-74.6
MC727b	5'cc**r**aaacccccc**r**cccg	Tm 61.6-70.5
MC851a	5'tgtttttgttgaatttttt**y**g**y**gggc	Tm 65-71
MC851b	5'c**r**c**r**aaaaaattcaacaaaaacaccg	Tm 63.5-69.6

The high density of CpG units in the promoter made it impossible to create primers free of them. Consequently, since the C in the units may or may not be methylated in the patient samples, we had to build this uncertainty into the primer sequences. The "y" and the "r" stand for "C or T" and "G or A", respectively. The melting temperatures will be effected, that is why we used a range in the Tm calculations.

First we tested and optimized the methylation specific primer pairs. For positive control, we used fully CpG-methylated human DNA from Intergene Inc. as template. Our testing DNA was the DNA preparation from the LNCaP-TJA prostate cancer cell line. These cells were predicted to have methylated APRIN promoter, since this gene appeared to be silenced and the cells did not arrest proliferation by androgens (3). To detect promoter methylation, both DNAs had to be converted by bisulfite-mediated oxydative deamination (8). We used the CpGenome DNA Modification Kit from Chemicon and followed the instructions in the kit. Typically, we converted 1 ug DNA in 100 ul starting volume and the conversion took place at 55 C^{o} overnight.

For the MSP PCR reactions we used 70-100 ng converted DNA. To find the optimal enzyme-buffer combinations we tested a variety of PCR systems specific for high GC amplifications. We tried the TripleMaster kit from Eppendorf with the10xHigh Fidelity buffer. We also tried the PCRx Enhancer system with the Platinum Taq DNA polymerase. Finally, we tried the Extensor Hi-Fidelity PCR System with Extensor Buffer 2 from the ABGene company. We found that the best results were generated by the PCRx Enhancer system with the Platinum Taq DNA polymerase. The PCR protocol was similar to the one mentioned above: 98 C°-3min (denaturation); 94 C°-30sec, 55 C°-30sec, 72 C°-1min, 5 cycles (touchdown phase); 94 C°-30sec, 51 C°-30sec, 72 C°-30sec, 45 cycles, followed by 72 C°-3min. The products were run in 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The results are shown in Figure 5.

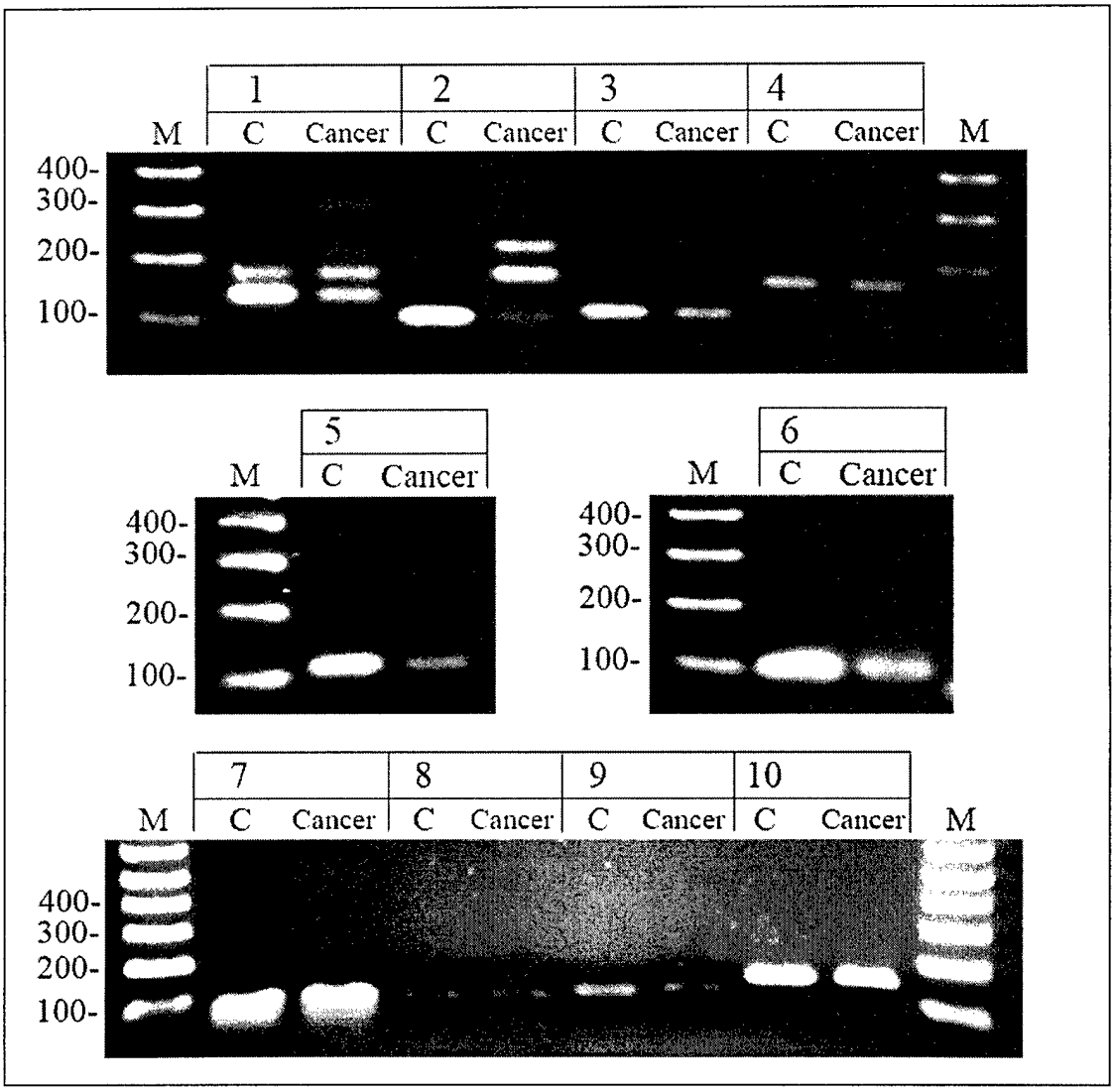

Figure 5 Methylation-specific PCR (MSP) test of the MC primer set. In lanes "C" (control), the template was the bisulfite-converted, fully methylated human DNA. In lanes "Cancer", the template was the bisulfite-converted LNCaP-TJA DNA. The following primer pairs were used: (mostly MC series; some MT primers were also used) 1, MTn38a-MC50b; 2, MC50a-MC133b; 3, MC133a-MC229b; 4, MC229a-MT400b; 5, MT400a-MT461b; 6, MT461a-MC536b; 7, MC536a-MC593b; 8, MC593a-MC650b; 9, MC650a-MC727b; 10, MC727a-MC851b. M, marker, in basepairs.

The results show that the primer design worked and most of the PCR reactions amplified the specific target sequences. We have already sequenced some of the amplicons and the specificity has been confirmed. These are the first results on promoter methylation in the TJA cell line and clearly show that the 15 CpG units our test analyzed are methylated.

Next we wanted to compare the methylation status of the APRIN promoter in the mother cell line LNCaP-FGC (where APRIN is inducible and the cells arrest) and in the TJA cell variant (APRIN is not inducible and the cells fail to arrest). We performed MSP reactions on the positive control DNA and bisulfite-converted DNA preparations from the FGC and TJA cell lines. The MSP reactions were performed with the MC50a -

MC133b primer pairs in PCR conditions described above. The results are shown in Figure 6.

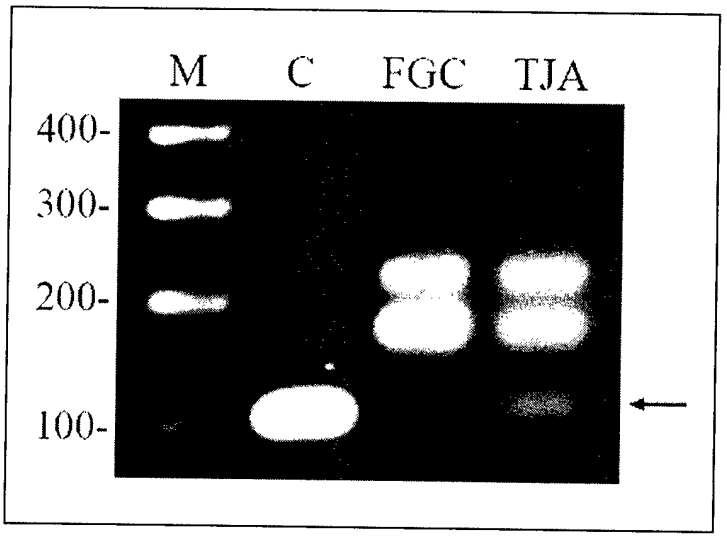

Figure 6. Methylation studies on the APRIN promoter in the LNCaP-FGC and LNCaP-TJA cell lines. Lane C, fully methylated positive control human DNA. Lanes FGC and TGA, MSP reactions on bisulfite converted DNAs from the FGC and TGA cell, respectively. The arrow indicates the APRIN promoter methylation specific band.

The results in Figure 6 indicate that the methylation-specific band (see lane C, positive control) is present in the TJA DNA but it is entirely missing from the FGC DNA. The data represent the first evidence that the development of the androgen-resistant phenotype of the TJA cells from the original FGC cells correlate with a change in promoter methylation status of the APRIN promoter.

We also performed bisulfite conversion and MSP reactions on the DNAs isolated from the paraffin embedded archive cancer samples (from Section 2) and from the needle biopsy materials in Section 3. The MSP reactions detected methylation in the DNA samples tested, as shown in Figure 7.

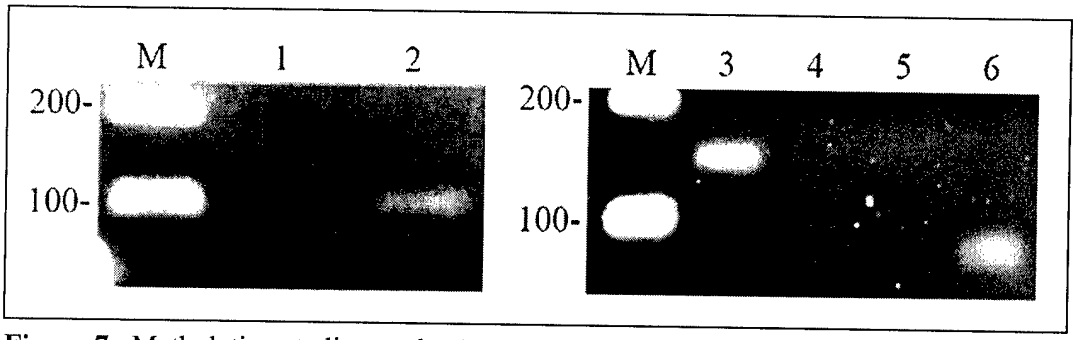

Figure 7. Methylation studies on the APRIN promoter of DNA samples from archive embedded tissue samples (lanes 1 and 2) and from needle biopsy samples (lanes 3-6). The following primer pairs were used; 1, MC536a-MC593b; 2, MT451a-MC536b; 3, MC727a-MC851b; 4, MC650a-MC727b; 5, MC593a-MC650b; 6, MC536a-MC593b. The results indicate the methodology is established to detect promoter methylation in minute amounts of clinical samples.

8) PCR amplify methylation-specific (bisulfite-treated) longer AS3 promoter elements (200-250 bp) for cloning and sequencing, to use in the global methylation analysis approach (from 3 post-operative samples and from cell lines LNCaP and MCF7). (Months 6-12). To amplify APRIN promoter sequences for direct sequencing analysis, we established a separate primer set. This set is specific for bisulfite-converted DNA, but does not target CpG units, so it amplifies DNA regardless of the methylation status. This primer set is shown in Table 3.

TABLE 3
APRIN promoter primers, to amplify bisulfite-converted DNA for sequencing

MTn38a	5′ gtygtaagagtagtggaygttttgatagttgtttt	Tm 62-67.6
MT50a	5′gyggggttgaggaggga	Tm 59-65
Mt50b	5′tccctcctcaaccccrc	Tm 59-65
MT133a	5′gtagggggygttgygtatttatgt	Tm 58-66
MT133b	5′acataaatacrcaacrccccctac	Tm 58-66
MT229a	5′ attttgaagyggagaggaggagg	Tm 62-66
MT229b	5′cctcctcctctccrcttcaaaat	Tm 62-66
MT400a	5′gaggtaggaaataatttagttaaygggaaagaaaatg	Tm 63.6-66
MT400b	5′cattttctttccrttaactaaattatttcctacctc	Tm 63.6-65
MT461a	5′gtgygtgtgtgtggtagggttgaag	Tm 65-69
MT461b	5′cttcaaccctaccacacacacrcac	Tm 65-69
MT536a	5′ ggtggttgtttggyggtggtt	Tm 64-68.4
MT536b	5′aaccaccrccaaacaaccacc	Tm 64-68.4
MT593a	5′ tgggggtggggagggtt	Tm 66
MT593b	5′aaccctccccaccccca	Tm 66
MT650a	5′ggttttgtygtagtttttggaaaattaaatttgt	Tm 65-67
MT650b	5′acaaatttaattttccaaaaactacracaaaacc	Tm 65-67
MT727a	5′ ggyggggggtttyggggtygt	Tm 65-75
MT727b	5′acracccccraaacccccrcc	Tm 65-75
MT851a	5′ggtgtttttgttgaatttttygygggg	Tm 65.3-71
MT851b	5′cccrcraaaaaattcaacaaaaacacc	Tm 65-71

We performed PCR reactions to optimize the primer pairs using the fully methylated human positive control DNA, in PCR conditions described above. The result in Figure 8 indicates that most of the primers worked and the methodology is available for the analysis of the methylation patterns by sequencing.

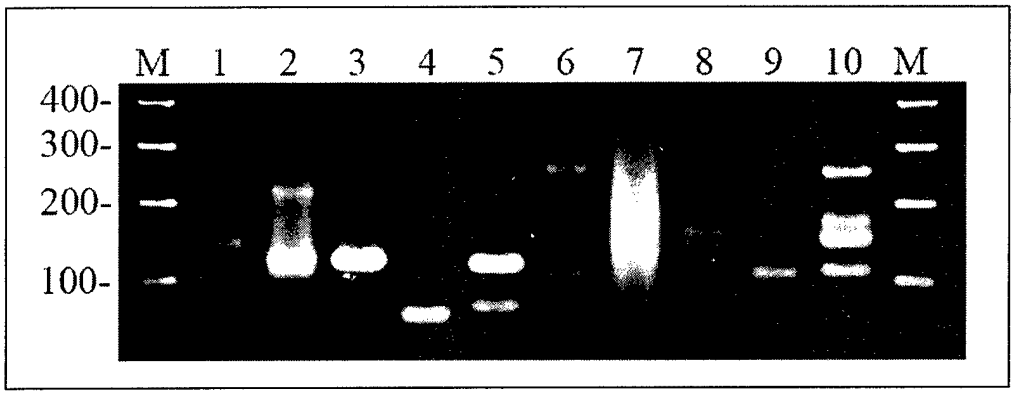

Figure 8. PCR reactions to amplify bisulfite-converted DNA for sequencing. The primer arrangements and the conditions are the same as in Figure 3.

Using the primer set shown in Figure 8, we performed PCR reactions on bisulfite-converted DNA from the LNCaP-TJA prostate cancer cell line. The PCR amplicons were isolated and sequenced directly, using the PCR primers. The advantage of the direct approach (instead of cloning and sequencing) is that the whole population is sequenced and the methylation ratios (the methylation percentage of any particular CpG units) can be directly quantified. Figure 9 shows the result of sequencing the MT50a-MT133b fragment.

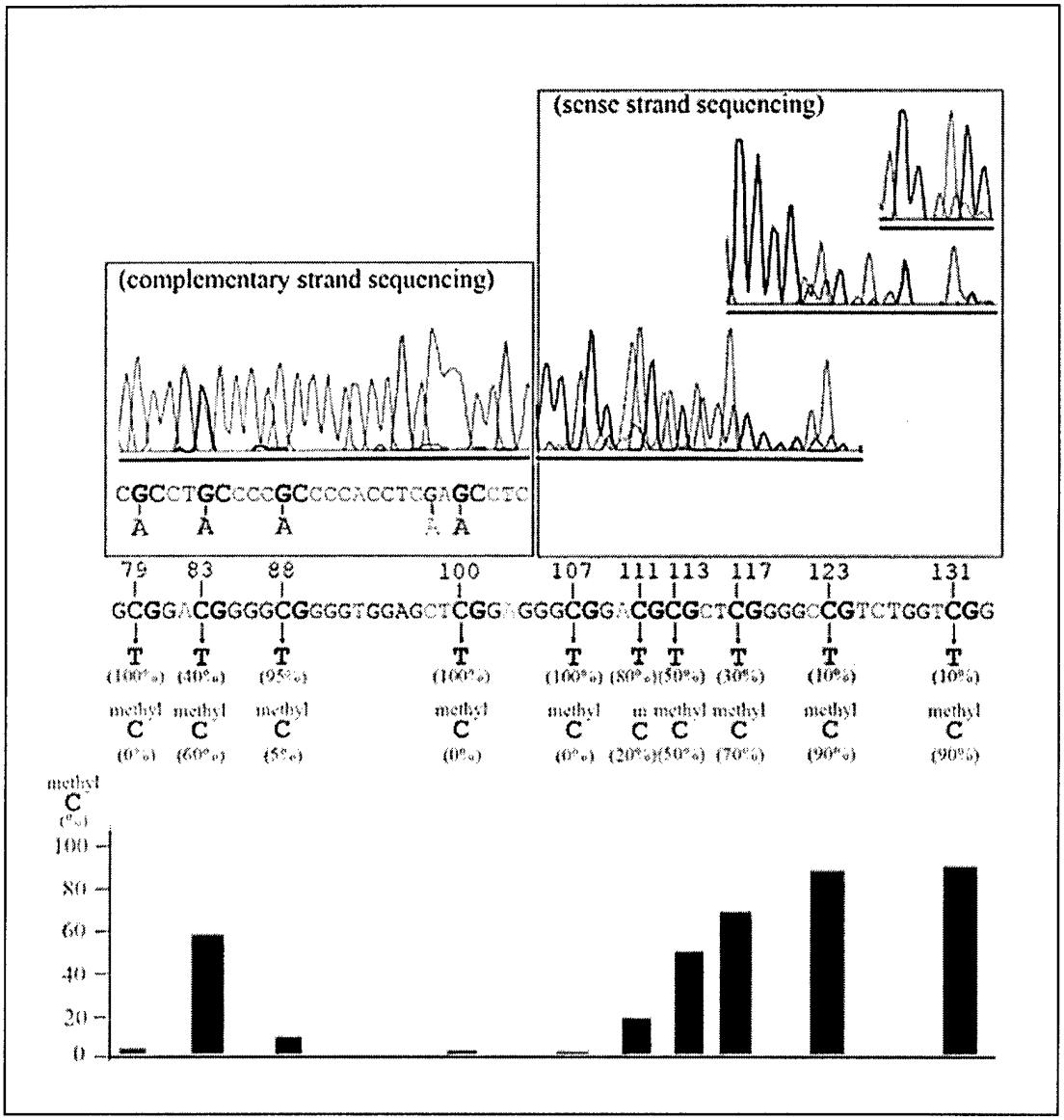

Figure 9. Methylation analysis on the APRIN promoter by sequencing. The top panels display the relevant portions of the sequencing printouts. The three graphs at the right show overlapping sequences in increasing resolutions. The nucleotide numbers correspond to the numbers in Figure 2. The C->T conversion ratios are directly indicated (under the "T"-s) The calculated methyl-C content is in blue and also shown in a graph format at the bottom.

The results directly confirmed CpG methylation in the promoter of the APRIN gene in the TJA cancer cell line. Highly methylated and non-methylated CpG units were identified. Similar date have been obtained from the other promoter areas.

9) Preliminary screening for informative methylated CpG targeting primers: characterize the range of methylation (methylation coefficient) in a small sample for each successful methylated CpG PCR target (using the above 10 postoperative samples, 20 archive samples and 10 needle biopsy samples, tumor and normal). (Months 6-24)
Some preliminary data are shown in Section 8 for this task.

10) Establish computer registry and record maintenance of samples (Months 1-24). we have only a limited number of samples so far, this task will be more important in the second year of the project.

KEY RESEARCH ACCOMPLISHMENTS

1) Established the methodology for DNA extraction from various sources, including extremely minimal clinical samples .

2) Performed and optimized the chromatin immune precipitation methodology to detect overall methylation status.

3) Designed, synthesized and optimized a set of new primer reagents; successfully detected and amplified the very complex and GC-rich promoter area of the APRIN (AS3) gene, the target of our studies.

4) Designed, synthesized and optimized novel primer reagents for the direct detection of promoter methylation (MSP reaction). Established and optimized the bisulfite conversion methodology for the detection of methylated CpG units.

5) Using the MSP primers, the first data have been generated that methylation of the APRIN promoter is critical in the silencing of the gene and correlates with hormone resistance in the LNCaP-TJA prostate cancer cell line.

6) Direct sequencing of the bisulfite-converted amplified promoter elements established methylation hot-spots in the promoter of the APRIN gene.

7) Established the methodology to detect promoter methylation from minute clinical samples and archive materials; preliminary data indicate promoter methylation in prostate cancer.

REPORTABLE OUTCOMES

Geck, P., Maffini, M. and Shu Ling Liang (2004) The APRIN gene is downregulated in invasive cancers. Proc. Am. Assoc. Cancer Res. 45:1292

Oral presentation in the 95th Annual Meeting of the American Association for Cancer Research, 2004, March 27-31, Orlando, FL. The Abstract was published in the Proceedings of the American Association for Cancer Research, Vol 45, p1292. The presentation included promoter methylation and gene silencing data in prostate, breast and ovarial cancers.

CONCLUSIONS

We have reached a few important milestones in the first year of the project. By establishing the methodology for DNA extraction from extremely minimal clinical samples, by working out the bisulfite conversion technology and by establishing the basic primer reagents for various tasks along the project, we set the foundations for the tasks of the second year. The first results with the chromatin immune precipitation methodology offer an alternative way to study promoter methylation

By using the MSP primers, we generated the first data that methylation of the APRIN promoter is critical in the silencing of the gene and correlates with hormone resistance in the LNCaP-TJA prostate cancer cell line.

These data have been confirmed by direct sequencing of the bisulfite-converted amplified promoter elements. In addition, these data established methylation hot-spots in the promoter of the APRIN gene.

Overall, we achieved significant progress towards our goals, to establish an early diagnostic and prognostic marker system in the clinical management of prostate cancer.

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